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The Photomorphogenesis Regulator DET1 Binds the Amino-Terminal Tail of Histone H2B in a Nucleosome Context

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Summary

Light provides a major source of information from the environment during plant growth and development [1, 2]. Recent results suggest that the key events controlling light-regulated gene expression in plants are translocation of the phytochrome photoreceptors into the nucleus, followed by their binding to transcription factors such as PIF3. Coupled with this, the degradation of positively acting intermediates such as the transcription factor HY5 by COP1 and the COP9 signalosome appears to be an important process whereby photomorphogenesis is repressed in darkness (e.g., [3–8]). Genetic analyses in *Arabidopsis* and tomato have revealed that the nuclear protein DET1 also plays a key role in the repression of photomorphogenesis [9–11]. However, the function of this protein has remained a mystery. In a series of in vitro experiments, we provide persuasive evidence that DET1 binds to nonacetylated amino-terminal tails of the core histone H2B in the context of the nucleosome. Furthermore, we have utilized FRET (fluorescence resonance energy transfer) imaging with GFP variants to demonstrate this interaction within the nucleus of living plant cells. Given the dramatic photomorphogenic phenotypes of *det1* mutants, we propose that chromatin remodeling [12–14] plays a heretofore unsuspected role in regulating gene expression during photomorphogenesis.

Results and Discussion

To investigate whether DET1 could interact with chromatin, we first examined the binding of DET1 to isolated nucleosomes. Histone H1-depleted mononucleosomes were purified from chicken erythrocytes, bound to CNBr-activated sepharose, and used for binding assays with in vitro-translated DET1 proteins. Figure 1A shows that tomato DET1 was selectively retained on this resin, as was *Arabidopsis* DET1 (data not shown), whereas a protein with similar size and charge characteristics (firefly luciferase) was not. DET1 was eluted from mononucleosomes at a concentration of approximately 400 mM NaCl (Figure 1B), suggesting a fairly strong binding affinity.

To determine whether DET1 binds to a specific histone within the mononucleosomes, we performed competition assays by incubating a 20-fold excess of each purified histone with DET1 prior to binding on nucleosome-sepharose. Figure 1C shows that histone H2B was the most effective competitor.

To confirm these results, binding assays were performed with a histone-agarose resin, which contains all the histone proteins covalently bound to agarose beads. We found that plant DET1 was retained on the resin, was eluted at a concentration close to 400 mM NaCl, and was competed only with histone H2B (see Figure S1 in the Supplementary Material available with this article online).

To define the domains of H2B that DET1 is able to interact with, we tested binding to GST fusion constructs containing different regions of human H2B [15]. Human H2B was used at this stage because its domains have been much better defined than have plant H2B proteins. Equal concentrations of purified recombinant proteins were immobilized on glutathione-sepharose and were used in in vitro binding assays. As shown in Figure 1D, DET1 binds only to the N-terminal 32 amino acids of H2B. This corresponds exactly to the N-terminal tail of human H2B, which extrudes from the core nucleosome structure [14]. To confirm the significance of this binding, we verified that DET1 could bind to the amino-terminal tail of plant H2B (see Figure S2 in the Supplementary Material).

In order to define the region of binding to H2B within DET1, we made a series of deletion constructs of tomato DET1. The corresponding in vitro-translated proteins were then used in in vitro binding assays with the GST-H2B N-terminal tail. This analysis revealed at least two distinct H2B tail binding domains within DET1, one within the N terminus (amino acids 1–148), and one within the C terminus (amino acids 374–523) (Figure 1E). Because of the high level of similarity with *Arabidopsis* DET1, we predict that these domains are likely to be the same in the *Arabidopsis* protein, although this was not experimentally tested. These domains are no more highly conserved than are other regions in eukaryotic DET1 proteins [16].

Because all of the experiments described above have been performed with in vitro-obtained products, we verified that DET1 in planta could behave in the same manner. For this, we performed binding assays with nuclear extracts prepared from leaves of transgenic tomato plants expressing epitope-tagged tomato DET1 (Myc-TDET1). These extracts were then used for binding assays with the different resins, and binding was detected by Western blotting with a mouse monoclonal antibody against the Myc epitope (Figure 1F). Although these experiments were complicated by the fact that this construct commonly induced posttranscriptional gene silencing in the transgenic plants (data not shown), the results nonetheless indicated that plant-purified DET1 behaves in exactly the same way as the in vitro-translated protein (both with and without a Myc tag). Furthermore, experiments with other Myc-tagged proteins (e.g.,

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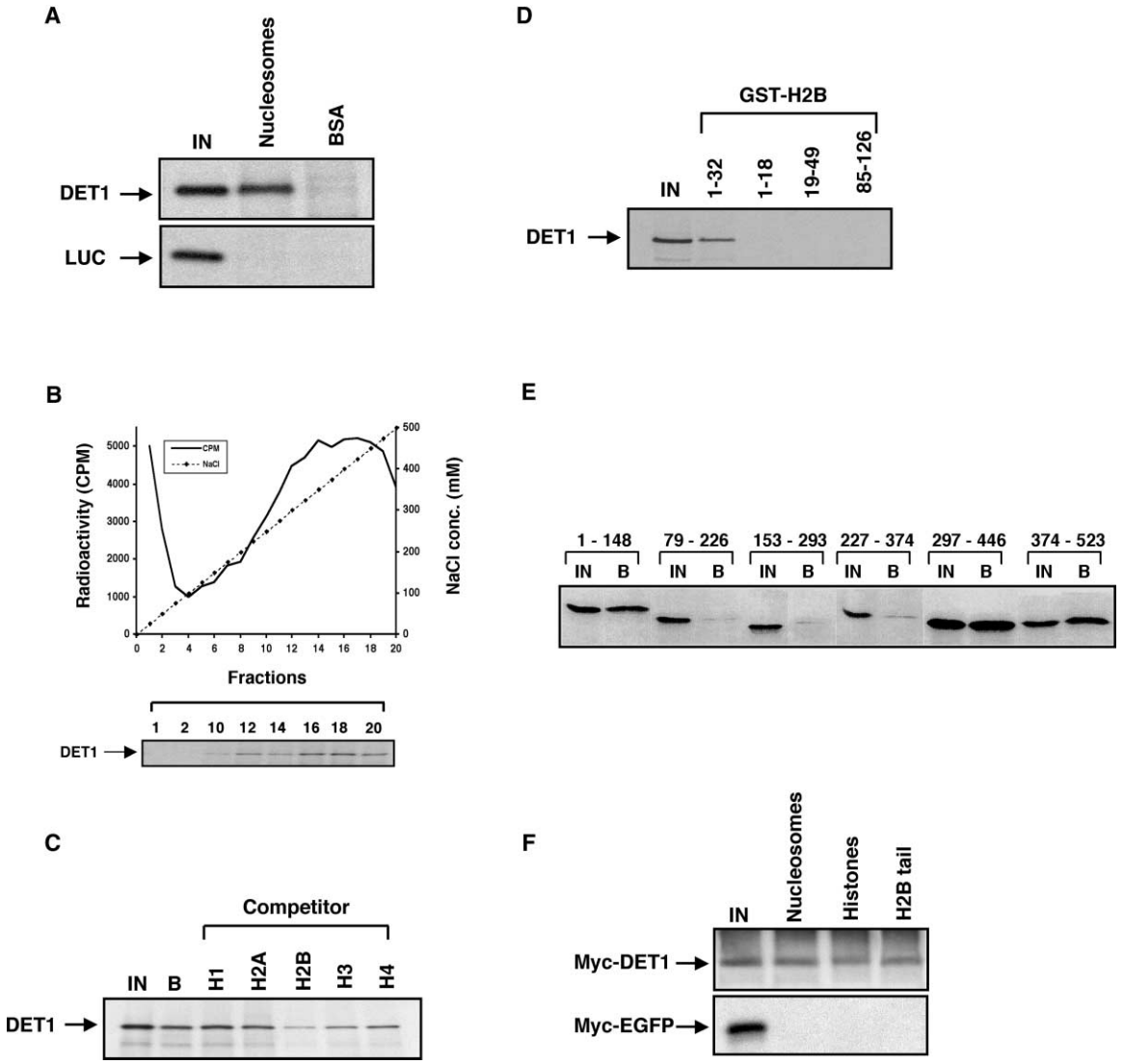

Figure 1. DET1 Binds the Amino-Terminal Tail of H2B in a Nucleosome Context

(A) DET1 binds mononucleosomes. An autoradiogram of binding assays of in vitro-translated tomato DET1 to nucleosome-sepharose or BSA-sepharose. In vitro-translated DET1 (3 μl) was incubated with 10 μl nucleosome- or BSA-resin in a final volume of 50 μl PBS + 1 mg/ml BSA overnight at 4°C with gentle agitation. The resins were resuspended in 15 μl 2× Laemmli buffer after washing three times with PBS. Bound proteins were resolved on 10% SDS-PAGE, and gels were then treated as described in the Experimental Procedures. In vitro-translated firefly luciferase (LUC) was used as a negative control for binding. Input (IN) is equivalent to one third of the amount used in the binding reaction.

(B) A chromatogram and autoradiogram of ^{35}S-labeled in vitro-translated tomato DET1 following elution from nucleosome-sepharose with a 10-ml linear 0–500 mM NaCl gradient in PBS. A nucleosome-sepharose column (5 mm i.d. × 10 mm) was connected to a FPLC unit and was run at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected, and 100 μl was used for liquid scintillation counting, while the remaining fraction was TCA/DOC precipitated, resuspended in 15 μl 1× Laemmli buffer, and subjected to SDS-PAGE. Following electrophoresis, the gels were treated as described in the Experimental Procedures.

(C) Binding of DET1 to nucleosome-sepharose is competed with histone H2B. A total of 100 μg of each purified histone protein (20-fold excess) was incubated with in vitro-translated tomato DET1 for 30 min before performing the binding assay to nucleosome-sepharose. The different histones used as binding competitors are indicated on top of each lane. As controls, one third of the total input (IN) and the bound fraction without competitors (B) are also included.

(D) DET1 binds the amino-terminal tail of histone H2B. GST pull-down assays were performed with different GST-H2B deletion constructs as matrices for binding. A total of 3 μl in vitro-translated tomato DET1 was incubated with 2 μg of each of the different GST-H2B fusion proteins immobilized on glutathione-Sepharose 4B. The bound material was then loaded on 10% SDS-PAGE and visualized by fluorography.

(E) At least two different domains within DET1 can interact with the H2B tail. In vitro binding assays (pull-down) of ^{35}S-labeled DET1 deletions to GST-H2B (amino acids 1–32) were performed as described in the Experimental Procedures. The different DET1 constructs are shown on top of the gel. For each in vitro-translated product, IN corresponds to one third of the total input, and B corresponds to the bound fraction.

(F) DET1 purified from plants interacts with histones. Western blot analysis of binding assays of nuclear protein extracts from Myc-TDET1 overexpressing tomato plants to nucleosomes, histones, and the N-terminal tail of histone H2B. Approximately 100 μg total nuclear protein extract was incubated with 10 μl nucleosome-sepaharose, 20 μl histone-agarose, or 20 μl GST-H2B (amino acids 1–32)-sepharose, in a final volume of 50 μl NEB, overnight at 4°C with gentle agitation. After washing three times with NEB, the resins were resuspended in 18 μl 2× Laemmli buffer. In vitro-translated Myc-EGFP was used to demonstrate that the Myc epitope tag did not influence binding. Bound proteins were resolved by 12% SDS-PAGE. The gel was then treated as described in the Experimental Procedures. Input (IN) is equivalent to one third of the amount used in the binding reaction.

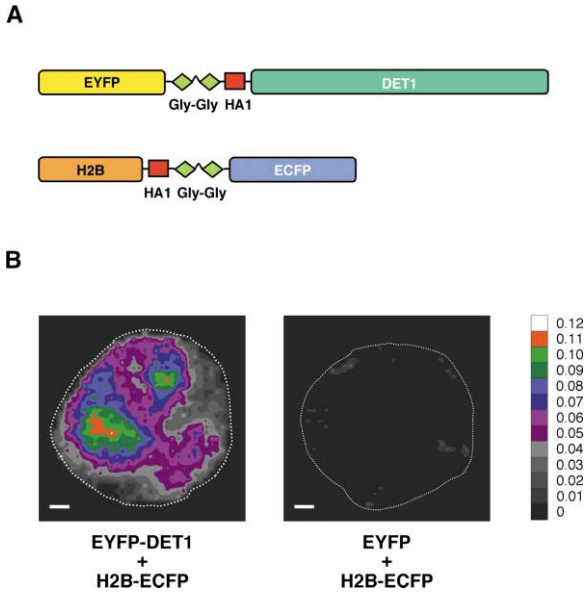

Figure 2. DET1 Binds Histone H2B In Vivo

(A) A schematic drawing of the two fusion constructs used for FRET experiments. DET1 and H2B were fused to the GFP variants EYFP and ECFP, respectively. A flexible linker constituted by an HA1 epitope tag and a Gly-Gly dipeptide was introduced between the two proteins used in each fusion.

(B) Representative FRETN' contour maps (and relative scale-color bar) obtained by calculating FRETN' pixel by pixel from the acceptor, donor, and FRET images of two nuclei (circled) of *N. benthamiana* cells cotransfected with the indicated constructs as described in the Experimental Procedures. Cells were analyzed by digital video microscopy 18 hr after transfection. The scale bar represents 1 μm.

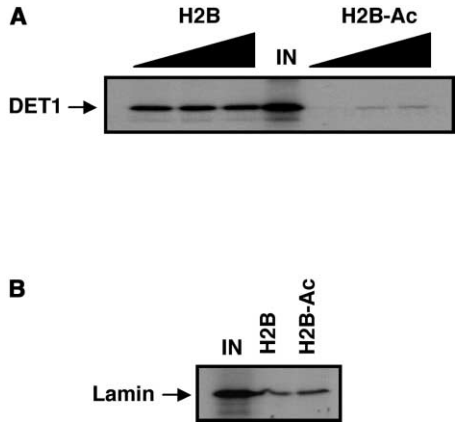

Figure 3. DET1 Binds Preferentially to Unmodified H2B N-Terminal Tails

(A) Peptides corresponding to the first 32 amino acids of H2B were synthesized either in the unmodified form (H2B) or were acetylated on lysines 5, 12, 15, and 20 (H2B-Ac). Increasing amounts of H2B N-terminal peptides coupled to amino hexyl-agarose were used as matrices for binding assays with 3 μl in vitro-translated tomato DET1. A total of 1 μl in vitro reaction mix (IN) was loaded to indicate the relative binding.

(B) A total of 3 μl in vitro-translated *Drosophila* lamin was bound to the unmodified (H2B) and acetylated (H2B-Ac) peptides. After several washes, the bound material was resolved on 10% SDS-PAGE and was visualized by fluorography. IN corresponds to one third of the total in vitro-translated product used in the binding assays.

Myc-EGFP) ruled out the possibility that the epitope tag had any influence on binding to the different resins (Figure 1F).

To observe the interaction between DET1 and H2B in vivo, we generated constructs in which tomato DET1 and tomato H2B were fused to green fluorescent protein (GFP) variants that could be used to measure the interaction between the two proteins by FRET (fluorescence resonance energy transfer) assays [17–19]. These fusions, denoted EYFP-DET1 and H2B-ECFP (Figure 2A), were generated in plant expression vectors and were transfected into cultured *Nicotiana benthamiana* cells by particle bombardment. In addition to EYFP-DET1/H2B-ECFP cotransfections, we performed three additional transfections (EYFP-DET1 and H2B-ECFP alone and, most importantly, H2B-ECFP plus EYFP, which would not be expected to interact), which were necessary for calibration and to exclude possible artifacts (see the Experimental Procedures in the Supplementary Material). Cells were screened 18 hr after transfection by digital epifluorescence microscopy. From each positive fluorescent cell, images in the acceptor, FRET, and donor channels were acquired, and the final FRETN' images were derived from these initial images [20] (see the Experimental Procedures in the Supplementary Material).

Figure 2B shows FRETN' images of representative cells cotransfected with EYFP-DET1/H2B-ECFP together with a negative control cotransfected with EYFP/H2B-ECFP. A significant difference was observed in the FRETN' signal within the nucleus of cells cotransfected with EYFP-DET1/H2B-ECFP compared with controls. The average FRETN' values for the test cells were more than 3-fold higher than those seen in negative controls (Table 1). These data demonstrate that DET1 can interact with H2B in vivo at a subnuclear level. Our images have not revealed any localized interaction within the nucleus but indicate that binding is generally diffuse throughout the nucleus.

Emerging correlations between gene expression and posttranslational modifications of the histone N-terminal tails have provided strong evidence that these modifications play an essential role in modulating the transcriptional activity within different chromatin domains [12, 13]. For example, acetylation of histone tails correlates with the activation of transcription. Because DET1 is a

Table 1. Statistical Analysis of FRET Data Derived from Nuclei of *N. benthamiana* Cells Cotransfected with Different Fusion Proteins

Transfected Fusions	Average FRETN' Value	Variance	t Test (P Value)	Number of Cells
EYFP-DET1 + H2B-ECFP	0.1528	0.0117	<0.004	13
EYFP + H2B-ECFP	0.0422	0.0020		13

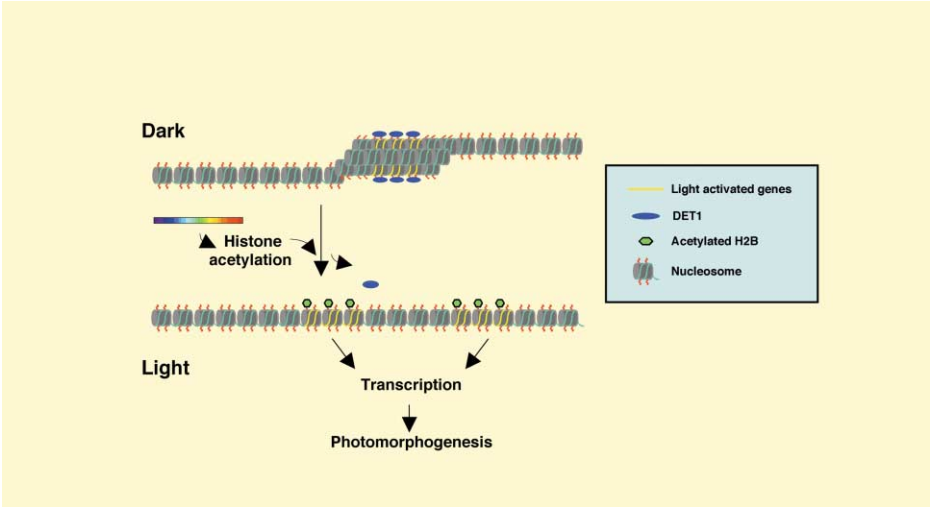

Figure 4. Model for Function of DET1

Based on the results of our experiments and on the phenotype of *det1* mutants, we propose a model for DET1 function. In dark (etiolated) conditions, light-regulated genes are not expressed and may therefore be localized within densely packed chromatin domains. DET1 binds to the nonacetylated H2B tails of the nucleosomes surrounding the promoters of these genes and maintains them in a repressed state. Light stimulation then leads to histone H2B acetylation (due to activation of an acetylase and/or deactivation of a deacetylase), followed by release of DET1, thus permitting transcriptional activation of genes involved in the de-etiolation response.

negative regulator of light-inducible gene expression, it was therefore of interest to test whether its binding to H2B tails could be influenced by acetylation. We used resin-immobilized synthetic peptides corresponding to the first 32 amino acids of human H2B in which the lysine residues had either been acetylated or not. These experiments revealed that DET1 binds preferentially to the unmodified form of H2B (Figure 3A). As a control, we used a *Drosophila* lamin construct [21] whose binding to H2B was not affected by acetylation (Figure 3B). Furthermore, we confirmed that the plant-derived Myc-DET1 protein also displayed preferential binding to nonacetylated H2B tails (data not shown).

The results presented in this report therefore provide suggestive evidence that DET1 binds to nonacetylated H2B tails within the context of the nucleosome. *Arabidopsis det1* mutants display a dramatic phenotype of constitutive photomorphogenesis in the absence of light [9], a consequence of the deregulation of hundreds of genes [22]. This information infers the importance of DET1 as a repressor of photomorphogenesis and implies that chromatin remodeling is an important event regulating the alterations of gene expression occurring during photomorphogenesis. Most simply, one could speculate that the chromatin context of photoregulated genes is characterized by nonacetylated histone tails and that the role of DET1 in the absence of photomorphogenic signals is to maintain this repressive chromatin state by interacting with unmodified H2B tails (Figure 4). Such a hypothesis is consistent with our knowledge of histone tail modifications in other eukaryotes, in which it has been shown that repressed regions of chromatin are characterized largely by the presence of unmodified histone tails [12, 13]. In such a scenario, the subsequent modification of H2B tails would relax the chromatin structure around photoregulated genes and permit their expression (Figure 4). Our results infer that acetylation of H2B tails may be an important step following light stimulation of dark-grown seedlings, although we cannot exclude the possibility that other modifications are involved.

Notwithstanding, the functional significance of DET1 interaction with H2B tails is unclear because it cannot be corroborated by mutant analysis. DET1 contains at least two autonomous domains that are important for H2B binding (Figure 1E), and, consequently, only a severely truncated protein will be unable to bind the H2B tail. *det1* mutants containing such mutations are likely to be null mutants [10] and so may mask other potentially important functional domains. Indeed, the *hp2* and *hp2^j* mutations in tomato *DET1* [11] do not affect binding to H2B in vitro (data not shown).

Studies of histone modifications in yeast and animal cells have led to the "Histone Code Hypothesis," whereby specific combinatorial modifications provide a "code" for the docking of different chromatin remodeling proteins [12, 13]. In particular, an enormous amount of research has demonstrated the importance of histone H3 and H4 tail modifications in the epigenetic control of gene expression in non-plant eukaryotic cells [12, 13, 23]. To our knowledge, DET1 in fact represents the first protein that specifically binds H2B tails. The presence of an expressed *DET1* homolog in the mouse and human genomes [11, 16] implies that it has a similar activity in other eukaryotes.

In line with studies in yeast and mammalian cells, one could predict the existence of a specific histone code that would signal chromatin remodeling around photoregulated genes. However, *DET1* is expressed at extremely low (albeit constitutive) levels in plant cells, and its subcellular distribution is not affected by light (unpublished data); so, it is unlikely that it can bind to any

nucleosome possessing an unmodified H2B tail. One possibility is that DET1 is only associated with nucleosomes around key photoregulatory genes (e.g., *HY5* and *PIF3* [5, 8]). A more complex histone code may therefore be necessary to recruit DET1 to nucleosomes associated with such genes; so, DET1 may not act alone. An independent study indeed provides evidence that DET1 is part of a multisubunit protein complex [22] (see below).

Mutation of the *DET1* homolog in *Drosophila*, known as *abo*, results in a maternal-effect lethality during early embryogenesis [16]. In corroboration with our results, Abo has been shown to bind to *Drosophila* chromosomes. Curiously, the protein localizes specifically to the histone gene cluster and negatively regulates their expression. Although these studies did not explore how Abo targets this chromosomal region, our results would predict that it does so via recognition of a specific histone code. It is unlikely, however, that DET1 targets histone genes in plants, because we have found no evidence that histone gene expression is deregulated in tomato *hp-2* mutants (unpublished data).

Our results also infer that histone acetyltransferases (HATs) and histone deacetylases (HDACs) are likely to play an important role in regulating changes in gene expression during photomorphogenesis (Figure 4). Reverse genetics approaches in which the expression of individual genes have been modulated have so far revealed only pleiotropic effects [24], although the fact that the *Arabidopsis* genome contains 12 *HAT* genes and 15 *HDAC* genes (www.chromdb.org) reveals that individual enzymes that target histones within specific nucleosome contexts are likely to be found. Notwithstanding, forward genetics screens for photomorphogenic mutants have so far failed to implicate a role for histone acetylation in light-mediated responses.

An independent study has provided evidence that *Arabidopsis* DET1 is part of a nuclear-localized complex of approximately 350 kDa [22]. One of the proteins within this complex has been identified as an *Arabidopsis* homolog of vertebrate DDB1 (UV-Damaged DNA Binding Protein 1), a subunit of the nucleotide excision repair enzyme that is defective in xeroderma pigmentosa-E patients [25]. Interestingly, in human cells, DDB1 has been found to be a component of the STAGA complex, a chromatin-acetylating transcripional coactivator that also contains GCN5, a histone acetyltransferase [26], and also a component of the TFTC HAT complex [27]. Although human DET1 has not yet been identified as part of these complexes, these results nonetheless provide further suggestive evidence for the association of DET1 with chromatin.

In summary, our finding that DET1 binds unmodified H2B tails within a nucleosome context, coupled with the dramatic phenotypes of *det1* mutants, infers that chromatin remodeling is likely to play an important role in the regulation of gene expression during photomorphogenesis. Most likely, DET1 may limit access of positive regulatory factors to the promoters of photoregulated genes (Figure 4). Further evidence for such a mechanism must be derived from studies of histone tail modifications within the nucleosomes associated with light-regulated genes. The availability of genome-wide analyses of photoregulated gene expression in *Arabidopsis* [3, 4, 22] may permit the identification of "hotspots" for chromatin remodeling within the genome. In parallel, identification of the genes to which DET1 is targeted via its association with H2B tails will reveal more information about the functional significance of this interaction. Utilization of the chromatin immunoprecipitation technique (e.g., [28, 29]) in *Arabidopsis* should allow these important questions to be addressed.

Supplementary Material

Supplementary Material detailing the Experimental Procedures and additional figures is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgments

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